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Review

Application of computer-assisted molecular modeling for immunoassay of low molecular weight food contaminants: A review

Zhen-Lin Xu[a,b], Yu-Dong Shen[a,b], Ross C. Beier[c], Jin-Yi Yang[b], Hong-Tao Lei[a,b], Hong Wang[a,b], Yuan-Ming Sun[a,b,*]

[a] College of Food Science, South China Agricultural University, Guangzhou 510642, China
[b] Key Laboratory of Food Quality and Safety of Guangdong Province, Guangzhou 510642, China
[c] Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, TX 77845-4988, USA

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ABSTRACT

Immunoassay for low molecular weight food contaminants, such as pesticides, veterinary drugs, and mycotoxins is now a well-established technique which meets the demand for a rapid, reliable, and cost-effective analytical method. However, due to limited understanding of the molecular structure of antibody binding sites and antigenic epitopes, as well as the intermolecular binding forces that come into play, the traditional 'trial and error' method used to develop antibodies still remains the method of choice. Therefore, development of enhanced immunochemical techniques for specific- and generic-assays, requires new approaches for antibody design that will improve affinity and specificity of the antibody in a more rapid and economic manner. Computer-assisted molecular modeling (CAMM) has been demonstrated to be a useful tool to help the immunochemist develop immunoassays. CAMM methods can be used to help direct improvements to important antibody features, and can provide insights into the effects of molecular structure on biological activity that are difficult or impossible to obtain in any other way. In this review, we briefly summarize applications of CAMM in immunoassay development, including assisting in hapten design, explaining cross-reactivity, modeling antibody–antigen interactions, and providing insights into the effects of the mouse body temperature on the three-dimensional conformation of a hapten during antibody production. The fundamentals and theory, programs and software, limitations, and prospects of CAMM in immunoassay development were also discussed.

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* Corresponding author at: College of Food Science, South China Agricultural University, Wushan Road, Tianhe District, Guangzhou 510642, China. Tel.: +86 20 85283925; fax: +86 20 85280270.
E-mail addresses: ymsun@scau.edu.cn, gzsyming@163.com (Y.-M. Sun).

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1. Introduction

Analysis of low molecular weight food contaminants, such as pesticides, veterinary drugs and mycotoxins, plays an important role in ensuring food safety. Due to the increasing number of contaminants in food, the implementation of effective safety programs requires analytical techniques that are cost-effective and capable of rapid response with high-throughput, and in some cases capable of working on-site or remotely on-line. Immunoassays based on antibody–antigen-binding properties are well established methods which fulfils the need for an analytical technique to help assess food quality and food safety that meets the prerequisite criteria. At present, many immunoassay screening methods have been successfully developed as alternatives to conventional instrumental or chemical methods for detecting low molecular weight food contaminants [1–5].

Generally, several important parameters should be considered in the development of an immunoassay, such as hapten/antigen design, type of antibody, required specificity, format, and the type of tracer to be used. The very first and key step is designing the hapten/antigen. Small molecules with masses less than about 1000–10,000 Da are usually not immunogenic [6]; therefore, they are required to be conjugated to a carrier protein (capable of generating an immune response) by using a linker or spacer arm. Research on hapten design and application to heterologous and homologous assays in synthetic pyrethroids was conducted by Lee et al. [7], and a review of approaches used to synthesize haptens used in immunoassay of organophosphate and synthetic pyrethroid insecticides was conducted by Skerritt and Lee [8]. Basic issues concerning structural aspects of hapten–antibody specificity in steroids were reviewed by Fránek [9]. There are a wide variety of conjugation chemistries available using carboxyl, amide, hydroxyl, sulfhydryl, carbonyl, or carbohydrate residues of the hapten, and the choice of which reactive group to use can be of vital importance in determining the specificity of the generated immune response [10]. Researchers have evaluated the effect on antibody production in respect to the heterology of spacer arm length. In general, it has been observed that spacer arm length of four atoms or more appears to have no or little effect on antigen recognition [11,12]. But heterology in the spacer arm structure of the coating antigen has produced remarkable improvement in a number of enzyme-linked immunosorbent assays (ELISAs) [12–15]. Heterology in the hapten structure in the immunogen (immunizing agent) compared to the hapten structure in the plate coating conjugate has also worked well in ELISAs [16,17]. Also, the conjugation position on the hapten has worked well in producing ELISAs [14,18], and it was found that the degree of hapten heterology paralleled the degree of ELISA sensitivity [14]. However, heterology may not be necessary in the coating conjugate if the hapten is large enough and the determinant group(s) (that part of an antigenic molecule against which a particular immune response is directed) is a long distance from the carrier protein [19]. Szurdoki et al. [20] has also reviewed factors that should be considered when setting up an immunoassay, and illustrated general principles and useful techniques that may be employed.

Today, development of immunoassays is still primarily based on trial and error. For a simple analyte, the hapten design may be predictable, but for many analytes with complex structures, the hapten design may be often unpredictable, and the results of a proposed hapten design must be tested by time-consuming animal experiments. For example, in order to generate a desired antibody with high affinity and specificity, a series of immunogens were designed and used to immunize animals, and then ELISA methods were used to screen for the best antibody [21–24]. These procedures are time-consuming and very laborious, and sometimes the obtained antibodies lack the required features to develop a useful immunoassay method. Newman and Price [10] pointed out that an understanding of the antibody binding site, the epitope (A localized region or site on the surface of an antigen that is recognized by the immune system, B-cells or T-cells, and is capable of eliciting an immune response and of combining with a specific antibody to counter that response.), and the intermolecular forces of the antibody–epitope interactions are fundamental to the design of immunoassays. This is of particular importance when developing antibodies to small molecules, because small changes in hapten structure can result in large changes in binding affinities [10]. In order to develop immunoassays that have better specificity and are more economical, the immunochemist must turn to novel approaches that are able to provide useful information regarding the physical/chemical properties of analytes and offer assistance in hapten design [25]. Computer-assisted molecular modeling (CAMM) [26] is one of these approaches.

CAMM has successfully been applied in many fields such as biology, pharmaceutical sciences, and biotechnology [26–29]. The use of CAMM can provide insights into molecular structure and biological activity that are difficult or otherwise impossible to obtain. Recently, it has been demonstrated that CAMM was one of several useful tools that helped immunochemists develop anti-hapten antibodies with desirable properties. Many immunoassays used for detection of low molecular weight food contaminants such as an algaecide [30], antibiotics [6,16,31–35], cork taint [36], herbicides [37–40], mycotoxins [41,42], pesticides [43–48], veterinary drugs [49–51] and other chemicals [52–56] have been developed with the use of CAMM methods (Table 1). CAMM can be used to assist in hapten design, study antibody–antigen recognition in cross-reactivity studies and model antibody binding sites (Table 1). However, CAMM results do not always correlate with the expected experimental results. Obviously, the complex immune system of a living animal is only partially understood. In this review, we discuss the fundamentals and theory of CAMM in immunoassay development, commonly used programs and software, examples of how CAMM was used to help in immunoassay development, and how and why the expected results are not always obtained and sometimes remain unexplainable.

2. CAMM fundamentals and theory

2.1. Fundamentals and theory

The origin of computational chemistry was driven by the desire to understand the relationship between structural features and observed properties [57]. Computational chemistry programs allow scientists to generate and present molecular data including geometries (bond lengths, bond angles, and torsional angles), energies (heat of formation, activation energy, etc.), electronic properties (moments, charges, ionization potentials, and electron affinities), spectroscopic properties (vibrational modes and chemical shifts) and bulk properties (volumes, surface areas, diffusion, viscosity, etc.) [58]. A variety of methods including molecular mechanics, *ab initio* quantum chemical methods, semi-empirical quantum chemical methods and density functional theory (DFT) are used for specific modeling situations. The principles and application of computational chemistry and molecular modeling were reviewed in detail by Ramachandran et al. [59].

When using molecular modeling to generate molecular data, the first step is to draw the molecule in the molecular modeling software and then perform an initial optimization. The minimum energy conformations are then determined. This is often accomplished using Allinger's standard MM2 force field [60]. A molecular modeling program such as CAChe (Fujitsu Management Services of America, Inc., Sunnyvale, CA, USA) introduces force field parameters

Table 1
Applications of CAMM[a] used for developing immunoassays for small molecular weight compounds.

Category of contaminant		
Target molecules	Purpose of CAMM studies	Refs.
Algaecide		
Irgarol 1051	Hyperchem software was used to evaluate the theoretical geometries and electronic distributions of the target and haptens, one hapten mimicked the target well and was chosen for immunizing.	[30]
Antibiotics		
Ceftiofur	CAChe software with the MM2 force field was used to calculate the minimum energy conformations of all structures. Molecular orbitals were calculated with the Alvarez collected parameters, the electrostatic potentials were superimposed on the 3D[b] isosurfaces, and hapten design, CR[c] and Ab[d] recognition studies were conducted.	[31]
Fluoroquinolones	The Sybyl program was used to obtain CoMFA[e] models for studying the quantitative structure-activity relationship between the Ab and all targets at the 3D level.	[32]
Kanamycin and Tobramycin	Chemoffice software and the Gaussian program were used to generate minimum energy conformations and electrostatic potentials of the target and analogs to help explain CR and study Ab recognition.	[33]
Sarafloxacin	CAChe software was used to obtain minimum energy conformations and electronic properties of the analyte and analogs to help explain CR and determine which structural features were important to antibody binding.	[34]
Sulfadimethoxine	CAChe software was used to compare the structural and electronic properties of various sulfonamides and to provide insight into potential mechanisms of Ab recognition.	[16]
Sulfonamides	CAChe software was used to obtain 3D structures and electronic properties of haptens and sulfonamide drugs to help explain CR and study Ab recognition.	[35]
Sulfonamides	QUANTA software was used to investigate minimum energy conformations and electrostatic potentials of targets, and to suggest generic haptens for producing broad-specificity antibodies.	[6]
Cork taint		
2,4,6-Trichloroanisole	Hyperchem software was used to evaluate the theoretical geometries and electronic distributions of target and potential haptens, and to predict the suitability of a particular hapten structure used for immunizing.	[36]
Herbicides		
Atrazine	The Insight II program was used to construct a 3D model of a monoclonal Fab[f] fragment and dock with the hapten to study the binding site of the antibody fragment.	[37]
Diuron	Initial Fab models were constructed from structure homology to known Abs with AbM software on a Silicon Graphics R-4000 work station. AbM identifies CDR loops with a canonical structure and selects coordinates. The stereochemical quality of the AbM model was analyzed with PROCHECK[g]. The model was visualized and changed with INSIGHT[h]. The Fab model was analyzed by superimposing it upon variable region domains of known antibody crystal structures.	[38]
Metamifop	CAChe program was used to obtain lowest energy conformers and electrostatic potentials of analyte, hapten and related ligands to help explain CR and study Ab recognition.	[39]
Triazines	Hyperchem software was used to obtain electronic distributions and molecular volumes of analytes and haptens. Based on the obtained data, distribution maps were obtained with PCA[i] (an unscrambler program) that compared the calculated result with experimental data.	[40]
Mycotoxins		
Aflatoxin M_1	CAChe software was used to obtain minimum energy conformations and electronic properties of analyte and analogs to help explain CR and understand antigen–antibody interactions.	[41]
Fumonisin B_{1-3}	CAChe software was used to obtain 3D lowest energy conformations of targets and analogs to help explain CR and study Ab recognition.	[42]
Pesticides		
Benzoylphenylurea	Spartan software was used to generate minimum energy conformations and PowerFit software was used to fit the hapten with the targets to help explain CR and study Ab recognition.	[43]
Parathion	Hyperchem software was used to obtain electronic distributions of the target and haptens, PCA (SPSS13.0[j] program) was then used to choose several haptens that were close to the target in the distribution map for potential immunizing haptens.	[44]
Parathion	The ProMod program was used to construct a 3D model of a scFv[k] fragment and dock it with the hapten to identify key contact amino acid residues that would be candidates for site-directed mutagenesis.	[45]
Permethrin	Chemoffice software was used to optimize geometries of haptens and the analytes, haptens that had matched geometries with the analytes were chosen for the immunizing haptens.	[46]
2,4,5-Trichlorophenol	Theoretical geometries and electronic distributions, as well as pK_a values were calculated by Hyperchem software and ACD/pK_a software, respectively, to compare the differences between the analyte and haptens. The hapten that best mimicked the analyte was chosen for the immunizing hapten.	[47]
2,4,6-Trichlorophenol	Theoretical geometries and electronic distributions, as well as pK_a values were evaluated by Hyperchem software and ACD/pK_a software, respectively, to compare the differences between the analyte and haptens. The hapten that best mimicked the analyte was chosen for the immunizing hapten.	[48]
Veterinary drugs		
Furosemide	CAChe software was used to optimize structures and determine electronic properties of the target and analogs to explain the CR and study Ab recognition.	[49]
Nicarbazin	CAChe software was used to generate structural configurations and electrostatic potential isosurfaces of haptens and targets to explain why a previously designed hapten failed to generate antibodies with desired properties, and to suggest a more rational hapten design.	[50,51]
Other chemicals		
Nitroaromatic	MOBY and SCHAKAL software programs were used to investigate the influence of the spacer arm on the haptens' conformation and electronic nature compared with the analyte, and to select suitable molecules for immunization.	[52]
Nonylphenol	Hyperchem software was used to evaluate the relationship of theoretical geometries and electronic distributions between target and haptens, and to select an immunizing hapten. The influence of introducing a spacer arm on the structural conformation of the hapten was also studied by molecular modeling.	[53]
Semicarbazide	The Gaussian program was used to calculate atomic charges and electrostatic potentials of the analyte and hapten to confirm the rationality of hapten design.	[54]

Table 1 (*Continued*)

Category of contaminant		
Target molecules	Purpose of CAMM studies	Refs.
Trinitrophenyl	The 3D-coordinates were generated by the SWISS-MODEL server, 3D-models of the CDRs[l] suggested that the hapten-interacting structure of the salmon antibody site was similar to the mammalian antibody site. The template used for 3D-modeling of the scFv clones was an X-ray structure of a mouse Fv-molecule. Electrostatic surface potentials were contoured using GRASP[m].	[55,56]

[a] Computer-assisted molecular modeling (CAMM).
[b] Three-dimensional (3D).
[c] Antibody (Ab).
[d] Cross-reactivity (CR).
[e] Comparative molecular field analysis (CoMFA).
[f] Antigen-binding antibody fragment (Fab).
[g] PROCHECK is software that checks the stereochemical quality of protein structures, Cambridge, UK; thornton@ebi.ac.uk (PROCHECK).
[h] INSIGHT 2.2.0 is a graphics program, Biosym Technologies, San Diego, CA (INSIGHT).
[i] Principal component analysis (PCA).
[j] SPSS is a software program, Chicago, IL, U.S.A. (SPSS13.0).
[k] Single-chain variable fragment (scFv).
[l] Complementarity determining region (CDR).
[m] GRASP is software for Silicon Graphics for graphical representation and analysis of structural properties, and is supported by funding from the National Science Foundation Grant#DBI-9904841, supplied free, developed in the Honig Lab, Columbia University, New York, NY; for questions write: grasp_info@flash62.bioc.columbia.edu (GRASP).

for all cases not addressed in MM2. Then the electronic wavefunction for each compound is calculated to determine the electronic properties by solving the Schrödinger equation using extended Hückel approximation [61]. These calculations are accomplished with software provided in the CAMM programs. The derived wavefunction data is converted into three-dimensional coordinates, which are used for visualizing electron densities and electrostatic potentials.

Immunoassays are based on binding properties of the antibody and antigen. The steric criteria and interactions resulting from electronic properties are thought to be mainly responsible for antigen–antibody recognition [40,44]. The lowest energy conformation, electrostatic potentials, volumes, as well as atomic charges of the target and the designed haptens can be obtained by the *ab initio* quantum mechanical model [40], DFT [54] or the semi-empirical model [47,48]. After comparison of the target's molecular properties with the molecular properties of the designed hapten(s), the hapten that best mimics the target is chosen as the immunizing hapten. It is suggested that the potential haptens are modeled with the attached spacer arm. Combining the spacer arm and hapten in the calculation will help the immunochemist determine potential effects that the spacer arm or conjugation chemistry might have on the hapten(s), and therefore, may further help the immunochemist choose the best immunizing hapten.

When studying cross-reactivity and antibody recognition, the three-dimensional conformations and electrostatic potential isosurfaces are most often generated for the analyte of interest, hapten, and other competitors, and then compared to determine what structural features contribute to antibody recognition [33–35]. The electrostatic potential isosurface results from coloring the electronic density surface according to the calculated electrostatic potential values. The electrostatic potential at a point near a molecule is the potential energy of a proton placed at that point. Molecular mechanics and quantum mechanics methods software supplied in the different CAMM programs are used to generate the three-dimensional conformations and electrostatic potential isosurfaces of the molecules being compared.

The three-dimensional quantitative structure–activity relationship (3D-QSAR) method can be useful in analyzing cross-reactivity data, and used to study antibody recognition with a comparative molecular field analysis (CoMFA) model [32]. CoMFA uses the steric and electrostatic fields generated for each molecule to build a pharmacophore model for a training set of molecules by specifying alignments and conformations of each molecule consistent with binding to a common receptor site. However, a latter extension referred to as comparative molecular similarity indices analysis (CoMSIA) uses five physicochemical properties, those properties used to calculate the CoMFA plus hydrophobic and hydrogen bond donor and acceptor properties [62–64]. The 3D-QSAR models may be valuable tools for guiding the rational design of haptens and for predicting activity prior to chemical synthesis.

The structure modeling of a recombinant antibody (scFv or Fab fragment) can be based on the crystal structure obtained by X-ray crystallography technology [37], or it can be based on the knowledge-based protein modeling program [45]. More information concerning molecular modeling of antibody combining sites can be found in the review by Webster and Rees [65]. After producing the molecular model of the combining site, the hapten/antigen/analyte can be docked with the model using docking programs to identify key amino acid residues important for antibody–antigen recognition. These amino acid residues can then be the focus of site-directed mutagenesis in order to improve antibody affinity.

2.2. Programs and software

Since the molecular modeling industry began with the formation of two companies in 1978 [57], a number of commercial programs are currently now available for scientists. Molecular mechanics treats atoms and bonds as balls and springs, and researchers have empirically adjusted the parameters to mimic experimental results [66]. The Gaussian series of programs performs *ab initio* quantum mechanics [54], MNDO molecular orbital calculations [33,36,47,48] and PM3 molecular orbital models [36,39,40]. These calculations are used for semi-empirical quantum mechanics, and the MM2 force field [30,40,44] is used for molecular mechanics. However, AMBER is a better force field than MM2 for proteins and nucleotides [66]. CAChe [34,35,39,50,51], Chemoffice [33], Hyperchem [30,36,47,48,53], Insight II [37], and Sybyl [32] can all be used for modeling the three-dimensional conformations of molecular surfaces or for computational docking experiments. However, the specific description of these computational programs is constantly changing due to the rapid development of the computational industry. Although various programs and software are available, the immunochemists' intuition and training is important and necessary to interpret the results appropriately.

3. CAMM applications

3.1. Prediction of rational hapten design

The importance of careful hapten design during immunoassay development can never be underestimated by immunochemists [10,21–25]. However, in some cases, the seemingly rationally designed hapten may never produce an antibody that is active against the targeted molecule. Beier and Stanker [50] designed a hapten (hydrazone) of 4,4′-dinitrocarbanilide (DNC) (Fig. 1A) and coupled it to keyhole limpet hemocyanin (KLH). Mice were immunized to produce antibodies, but the antibodies obtained did not compete with "free" DNC. Through molecular modeling the researchers found that the hapten they first designed was both structurally and electronically different from DNC (Fig. 1A and B) [51]. Fig. 1C shows a comparison of a ball and stick model of free DNC and DNC with the attached hydrazone linker arm. Free DNC is planar, but following the addition of the hydrazone, a distinct angle is observed between the two phenyl-ring moieties of DNC in the hydrazone hapten. Following further molecular modeling studies, they proposed a DNC-mimic hapten, *p*-nitrosuccinanilic acid, to be used in the immunizing conjugate. As a result, anti-DNC monoclonal antibodies (MAbs) were obtained, which showed no cross-reactivity to the non-active component, 2-hydroxy-4,6-dimethylpyrimidine (HDP), in the nicarbazin drug complex, but did show good cross-reactivity with DNC (note added in proof: another laboratory [67] tried using different DNC mimics to produce antibodies without the help of molecular modeling, but found that the best hapten was the DNC mimic suggested by Beier and Stanker, who discovered that mimic through the use of CAMM [51]). The MAb with the best detection limit had an IC_{35} value of 0.92 nmol mL^{-1} for DNC, and was a good candidate for use in a competitive ELISA (cELISA) for the determination of nicarbazin in animal feeds [13]. In this immunoassay the IC_{35} was used because the high quantity of organic solvents required in the assay depressed the standard curve. The IC_{35} was located at the central point of the standard curve [13].

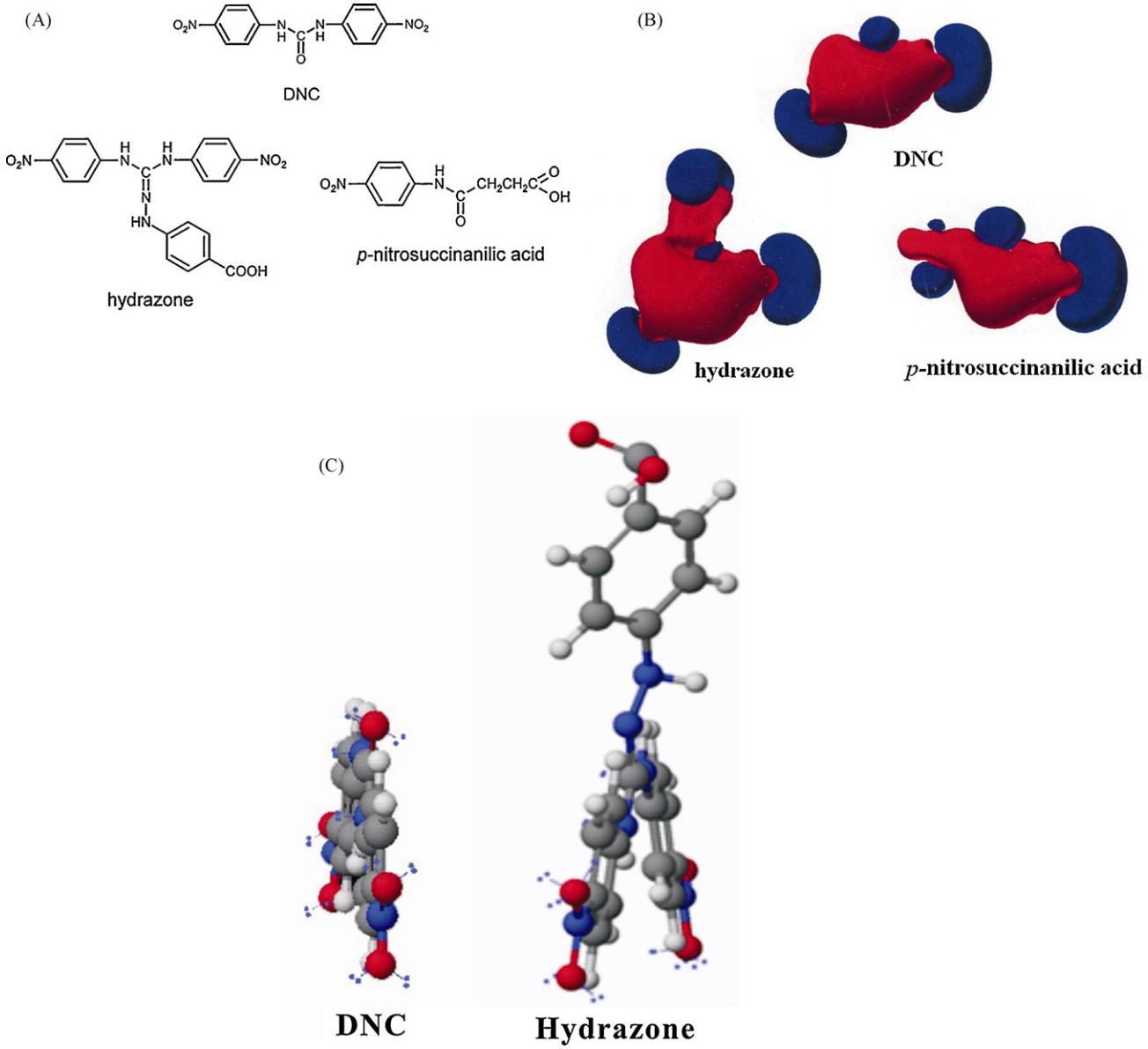

Fig. 1. Chemical structures (A), electrostatic potential isosurfaces of the molecular models of DNC and the two haptens, hydrazone and *p*-nitrosuccinanilic acid (B), and a ball and stick model comparison of 4,4′-dinitrocarbanilide (DNC) and DNC with the hydrazone linker arm (hydrazone) (C) [50,51].

Small molecules that are generally too small to induce an immune response are conjugated to a carrier protein by using a linker. However, introduction of a linker or spacer arm between the hapten and carrier protein may cause perturbations in the three-dimensional conformation of the hapten as well as concomitant changes in its electronic distribution. Galve et al. and others used CAMM and theoretical calculations to study the effect of introducing a spacer arm at different benzene ring positions on chlorophenol derivatives [36,47,48]. They found that the introduction of a spacer arm at different positions on the benzene ring did not produce significant conformational changes. The differences between the various haptens and the target analytes were observed in the molecular electronic distributions, especially with the acid–base equilibrium of the phenolic compounds in aqueous media. The best hapten candidate was chosen after calculating the theoretical geometries and electronic distributions, along with further theoretical calculations regarding pK_a values related to deprotonation enthalpy (DPE) and formation enthalpy (ΔH_0). As a result, highly sensitive and reliable analytical immunochemical techniques for 2,4,6-trichlorophenol (IC_{50} of $2.76 \pm 0.26\ \mu g\ L^{-1}$), 2,4,5-trichlorophenol (IC_{50} of $0.6\ \mu g\ L^{-1}$) and 2,4,6-trichloroanisole (IC_{50} of $0.19\ \mu g\ L^{-1}$) were developed. In the development of an ELISA for detection of nonylphenol (NP) [53], the use of CAMM suggested that either the ortho or meta positions of the phenol appeared appropriate for linking the hapten, 5-(2-hydroxy-5-nonylphenyl)-pentanoic acid (NPVA), to a carrier protein. A four-carbon atom alkyl-chain spacer arm was proposed to link the immunizing hapten to lysine residues of the horseshoe crab hemocyanin. CAMM was used to ensure that no significant changes in the geometry and electronic properties of the hapten, compared to the target analyte, would be introduced by adding a spacer arm at the ortho-position. They also used CAMM to determine that the availability of the phenol group for interaction with the antibody was fully preserved, and the results obtained showed that NP and NPVA had identical geometries at their minimum energy level (Fig. 2). The calculated root-mean-square error (RMSE) value was only 0.127 Å, which demonstrated that the geometry was not significantly affected by introducing the spacer arm. However, the authors realized that the phenol group may actually reside closer to the protein, which then may allow the nonyl chain to be more favorably exposed to the immune system. They also realized that once the hapten was coupled to the immunizing carrier protein that the hydrophobic alky chain could be affected by the tertiary structure of the protein resulting in hydrophobic interactions with the carrier protein. The developed ELISA had a limit of detection (LOD) of $2.3 \pm 0.9\ \mu g\ L^{-1}$ and an IC_{50} value of $29 \pm 5\ \mu g\ L^{-1}$ [53].

CAMM was used in an iterative process to improve hapten design and improve antibody recognition [25]. Several sensitive and specific immunoassays have been developed for the determination of irgarol 1051, metamifop, parathion, permethrin, and semicarbazide (metabolite of nitrofurazone) with the use of CAMM methods [30,39,44,46,54]. In most of these studies, several haptens were designed as candidates and then CAMM was used to optimize the energy and calculate the valences and charges. The hapten which was both structurally and electronically most similar to the target analyte was selected as the immunizing hapten. Generally, the obtained antibody exhibited high sensitivity and specificity to the target analyte and low cross-reactivity with other analogs.

Recently, the development of immunoassays capable of measuring multiple targets during a single test have been investigated, and these immunoassays are called broad-specificity, generic, group- or class-specific, or multi-analyte assays [68–70]. For a class-specific assay, selection of a hapten consisting of those features common to all the structures within the group to be targeted is required. Several attempts have been made to develop broad-specificity immunoassays for sulfonamides [6,35], triazines [40] and nitroaromatic residues [52] using the help of CAMM. Muldoon et al. [35] were interested in developing sensitive, class-specific antibodies for sulfonamides and supported their cross-reactivity studies with molecular modeling. The traditionally produced MAbs did not exhibit a broad cross-reactivity pattern and did not bind the hapten used in the injected protein-conjugate. However, CAMM studies of the hapten discovered evidence of structural conformation-selective hapten recognition. The authors concluded that design of haptens for the purpose of generating cross-reactive antibodies should not just consider the two-dimensional structure, but also the three-dimensional conformation (discussed more detail in Section 3.4) [35]. Spinks et al. [6] used molecular modeling of sulfonamide structures to obtain a broad-specificity immunoassay; however, despite some recognition of different immobilized sulfonamides, the desired broad-specificity recognition of un-conjugated sulfonamides was not achieved. They used molecular modeling studies of sulfonamide structures to determine that the drugs each had a characteristic "bend" around the $-SO_2-$ group. Based on a hypothesis derived from structural bending two conjugates were generated, one contained the hapten, sulfacetamide (SAM, more planar) and the other contained sulfachlorpyridazine (SCP, more bent). There was no competition observed for the anti-SAM anti-

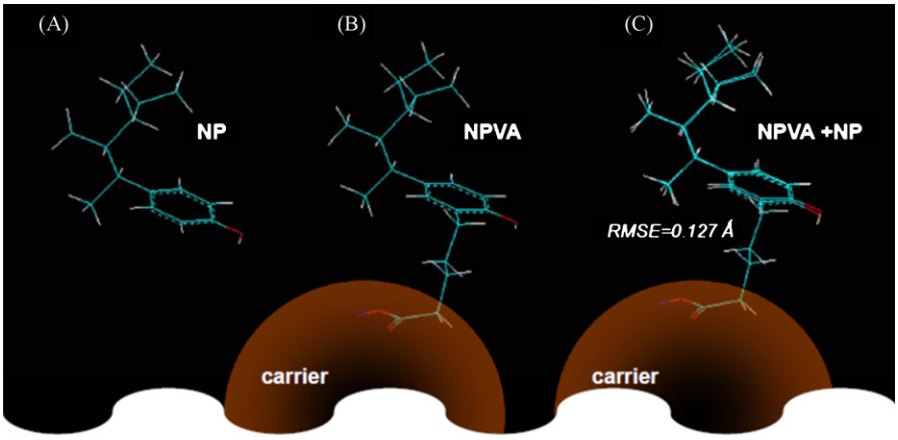

Fig. 2. Stick and wedge display of the optimized geometries of nonylphenol (NP) (A) and the hapten 5-(2-hydroxy-5-nonylphenyl)-pentanoic acid (NPVA) (B) according to PM3 models. The model (C) shows both compounds overlapped when differences in the geometries were calculated (RMSE = 0.127 Å). Calculations were made using the corresponding amide derivatives to mimic the conjugated haptens. The elements are presented in the following manner: light blue, carbon; dark blue, nitrogen; white, hydrogen; and red, oxygen [53]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

serum. Anti-SCP antiserum cross-reacted with some sulfonamides, and these appeared to have close structural similarity in the R-group [6]. Sprinks et al. [6] suggested that a limiting factor when using CAMM will be the poor understanding of hapten–antibody interactions relative to protein–antibody interactions. They concluded that CAMM studies of potential hapten structures revealed unexpected complexity among a family of related sulfonamide structures. Also, while CAMM was unable to solve the problem of producing broad-specificity antibodies in their situation, it did help illuminate new routes of experimentation [6]. It should be pointed out here that the use of CAMM cannot be expected to solve every problem. CAMM allows one to view the three-dimensional conformation and the electronic properties of the antigen and related compounds or a group of related compounds, then one can make an informed evaluation of the molecules based on the CAMM derived information. It is the immunochemist that must define the best hypothesis to proceed with using this information to guide the work that must be accomplished to arrive at the desired result. CAMM is only one tool that can help the immunochemist formulate a clear research direction and whether a project is successful or fails does not rest solely with the CAMM result. CAMM only can provide the opportunity for the immunochemist to view the three-dimensional conformations and electronic properties of the molecules of interest. Delaunay-Bertoncini et al. [40] used CAMM in a predictive manner in order to enhance the possibility of developing broad-specificity anti-triazine antibodies. Following determination of extraction recoveries of triazines using three different antibodies bonded to solid-supports, CAMM studies were conducted of the triazines, metabolites, and the involved immunoconjugates. Since antigen–antibody recognition is based on steric criteria and interactions resulting from the electronic properties of molecules, CAMM was used to determine molecular volume and the charges of compounds in combination with principal component analysis (PCA) to obtain distribution maps with the relative position of the three immunizing haptens and all the triazines. PCA involves a mathematical procedure (orthogonal linear transformation) that transforms a number of possible correlated variables into a smaller number of uncorrelated variables called 'principal components'. In all three cases, conclusions on specificity made with the analysis of the distribution maps fit well with the experimental results. They suggested that CAMM coupled with PCA was a unique, rapid and inexpensive tool to help select an appropriate hapten providing specific or class-specific antibodies according to the given problem. Jülicher et al. [52] used CAMM to investigate the structure of the hapten and evaluated the influence of the spacer arm on hapten conformation and electronic nature as compared with the analyte. A suitable molecule based on CAMM results was selected for use in immunization, and two polyclonal antibodies were raised; one was selective to 2,4,6-trinitrotoluene, and the other was able to recognize important structurally related compounds at low concentrations (2–1000 $\mu g\,L^{-1}$). Although there are few published examples, CAMM is believed to be a useful and potential tool in helping develop immunoassays with better sensitivity and broad-specificity [25]. Spinks et al. [6] suggested that the use of molecular modeling can help understand more about antibody–target interactions, and that molecular modeling will increasingly become a useful tool for the immunochemist.

3.2. Study of cross-reactivity and antibody recognition

Currently, most researchers use CAMM as a tool to help explain cross-reactivity of antibodies. Beier and Stanker used CAMM to predict antibody binding to target analytes and related compounds by comparing cross-reactivity data obtained from immunoassay competition studies with calculated minimum energy conformations and with either the electrostatic potential isosurfaces or with electron density isosurfaces of the binding and nonbinding molecules [71]. CAMM was used to evaluate antibody cross-reactivity results against the veterinary drug furosemide [49], and the antibiotics sarafloxacin [34] and sulfadimethoxine [16,35] in comparison with related analogs. These studies demonstrated that CAMM can aid in understanding what structural and electronic features are important for antibody binding, and it can help explain unexpected cross-reactivity results. Cross-reactivity is commonly a comparison between the target analyte and other molecules of interest to determine relative antibody binding, and then the cross-reactivity results are compared with the CAMM results to help understand antibody binding. Percent cross-reactivity is defined as (50% inhibition of control (IC_{50}) of the target analyte/IC_{50} of another compound) × 100. However, it was observed that cross-reactivity, calculated using IC_{50} values with units of weight per volume (e.g., $ng\,mL^{-1}$), as is often used, can result in incorrect cross-reactivity results for some compounds. But calculations using units of moles (e.g., $nmol\,mL^{-1}$) gave a consistent reliable result [13]. Cross-reactivity is not dependent on weight, but it is a site specific, structure specific phenomenon that is a molar dependent quantity. That is why the immunochemist can design a specific hapten to produce an antibody that specifically will bind an epitope on a target analyte resulting in a quantitative method. Therefore, when comparing target analytes to molecules of differing molecular weight, cross-reactivity should not be calculated using IC_{50} values with units of weight, but cross-reactivity should be calculated using IC_{50} values with units of moles (e.g., $nmol\,mL^{-1}$ or $pmol\,mL^{-1}$), as seen in following Refs. [13,15,72,73].

There can be great difficulty in developing generic immunoassays. The difficulty of developing a class-specific immunoassay for sulfonamide veterinary drugs was overcome by manipulating antibodies and using a combination of antibody cocktails plus heterologous ELISA formats [16]. A heterologous ELISA may use one chemistry, linker arm and hapten to produce the antigen used to immunize the host animal and a different chemistry, different linker arm or hapten to produce the plate coating antigen. CAMM studies of the cross-reactive drugs suggested that both steric and electronic features played a large role in antibody binding. Holtzapple et al. [41] examined binding of structural analogs to anti-aflatoxin M_1 MAbs, and correlated these binding characteristics with conformational and electronic properties of the analogs. The results demonstrated that in their study the loss of optimum structure and introduction of steric hindrance in the portion of the molecule that would fit into the antibody binding site was more important to binding than simply the loss of a determinant group [41]. The authors indicated that the information gained from CAMM can be used to explain the wide variation in IC_{50} values observed between structural analogs and as a tool for determining which conformational and electronic properties of molecules are most important for antibody recognition. During the development of an ELISA for fumonisin B_{1-3} (FB_{1-3}) the cross-reactivity of 14 anti-FB_1 MAbs were studied [42]. The results demonstrated that these antibodies recognized FB_{1-3} to varying degrees, and the hydrolyzed backbone of the fumonisins was recognized to a small extent. The use of CAMM explained the results by identifying the unexpected molecular folding of the amine backbone with the two esterified trimethylpropane-1,2,3-tricarboxylic acid side-chains on FB_1 [42]. The folding brought the expected epitope (esterified trimethylpropane-1,2,3-tricarboxylic acid) into close proximity to the nitrogen atom that was used to make the linkage to ovalbumin and bovine serum albumin. More antibody binding was observed to conjugated (less folded) structures rather than to un-conjugated (more folded) structures. CAMM made it readily apparent after viewing the three-dimensional conformation of FB_1 that the nitrogen atom on carbon 2 of FB_1 was not the most optimum site to attach the antigenic proteins required for antibody production.

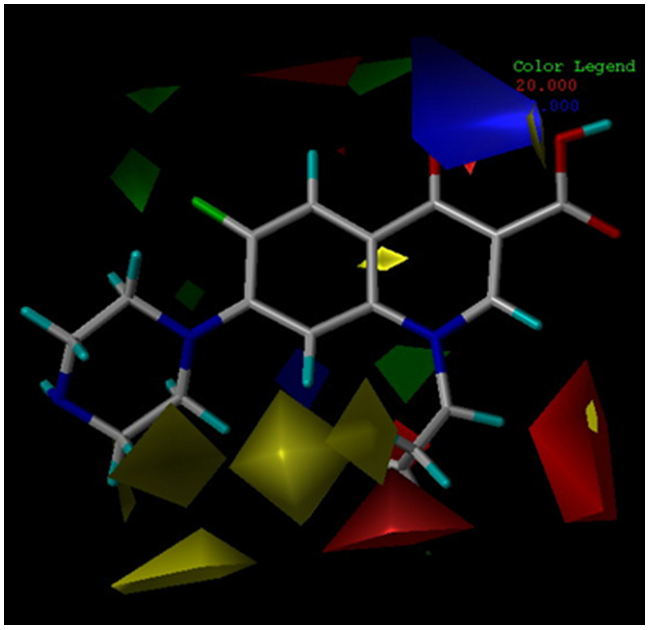

Fig. 3. Contour plots of the steric and electrostatic fields calculated using comparative molecular field analysis (CoMFA). Green contours indicate regions where bulky groups increase antibody affinity and yellow contours indicate regions where bulky groups decrease antibody affinity. Blue contours indicate regions where positively charged groups increase antibody affinity, and red contours indicate regions where negatively charged groups increase antibody affinity [32]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Wang et al. [43] developed a class-specific competitive ELISA for the benzoylphenylurea insecticides and used CAMM to examine the superior cross-reactivity of diflubenzuron. The results suggested that the planarity of the molecules, electron-withdrawing groups, and steric effects of the chlorines attached to the phenyl ring may be critical factors affecting antibody binding. Recently, Wang et al. [32] developed a broad-specificity MAb which exhibited high cross-reactivity (35–100%) with 12 fluoroquinolone (FQ) antibiotics. The CAMM results showed that the steric factor for groups or atoms played more of an important role in antibody affinity for the FQ analogs than did the electrostatic factor. Further, by developing a three-dimensional quantitative structure–activity relationship (3D-QSAR) using the CAMM method of comparative molecular field analysis (CoMFA), a structure–activity relationship model of the epitopes for 14 structurally related FQ analogs was constructed (Fig. 3). The model was useful in analyzing cross-reactivity data and it could also predict antibody binding to analogs that were outside of the study. Chen et al. [33] raised MAbs with high cross-reactivity and sensitivity to tobramycin (IC_{50} of 0.89 ng mL^{-1}) and kanamycin (IC_{50} of 0.83 ng mL^{-1}), and the MAbs had slight or negligible cross-reactivity with other aminoglycosides. The specificity and cross-reactivity of the antibodies were discussed regarding the three-dimensional molecular models of the aminoglycosides.

CAMM studies were conducted to compare structural and electronic properties of the target analyte and other related analogs and provided insight into potential mechanisms of antibody recognition. The use of rational hapten design based on structural and electronic features important for antibody binding have produced effective haptens for both specific assays and class-specific assays.

3.3. Modeling antibody- and antibody fragment–antigen interactions

Antibody–antigen interactions are fundamental to immunoassay. These interactions are comprised of hydrogen bonds, Van der Waals forces, hydrophobic interactions and electrostatic bonds [25]. Most often researchers can only study antibody–antigen-binding properties indirectly through experimental data such as IC_{50} and cross-reactivity values [16,34,40–43,71]. Based on successful applications of computer-assisted modeling technology in drug discovery and biology [27–29], CAMM has been used to help understand and predict molecular recognition, both structurally (three-dimensional) by finding likely binding modes, and energetically by predicting binding affinity [74]. The commonly used methods for studying antibody–antigen interactions are molecular docking and 3D-QSAR, such as CoMFA and CoMSIA methods [74–77].

The most commonly utilized antibodies in immunoassay are of the IgG isotype. These proteins are made up of four linked polypeptide chains, two identical heavy and two identical light chains (Fig. 4A) [78]. The three-dimensional models of the structures can be constructed by computer once the amino acid sequence has been identified (Fig. 4C) [29]. However, since antibody molecules are very large, structural modeling of antibodies requires sophisticated software running on powerful workstations [25]. Therefore, the antibody–antigen structural binding site is difficult to model. The use of recombinant antibody fragments (Fig. 4B) for detecting low molecular weight food contaminants [78–80] has overcome the difficulty of using CAMM for exploring antibody–antigen interactions, because the size of the recombinant antibody fragments are remarkably smaller than the natural antibody.

Kusharyoto et al. [37] constructed a three-dimensional model of the variable domain of an atrazine-specific antigen-binding antibody fragment (Fab) K411B using CAMM. The molecular dynamic simulations and cross-reactivity data were then used to predict the Fab fragment amino acid residues responsible for binding the hapten 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) (iPr/C1/C6) (Fig. 5). Kusharyoto et al.'s combined results from CAMM and site-directed mutagenesis allowed them to engineer a Fab fragment capable of recognizing the hapten better than the wild-type Fab fragment. They also identified residues responsible for spacer recognition. This provided the potential to increase the sensitivity of a competitive direct ELISA due to the decrease in relative affinity for spacer recognition of the hapten iPr/C1/C6. Chambers et al. [45] obtained a single-chain variable fragment (scFv) recombinant antibody against the organophosphate pesticide ethyl-parathion and used ProMod software to generate a molecular model of the ethyl-parathion-recombinant antibody interactions. Docking studies showed the involvement of the complementarity determining region (CDR) H_3, CDR L_2 and the small areas of the framework region of the light chain during binding. Bell et al. [38] modeled the diuron-recombinant antibody interactions to identify key contact amino acid residues that would be candidates for site-directed mutagenesis. The model offered a starting point for knowledge-based manipulation of the scFv with the aim of changing specificity to the organophosphates.

The modeling of antibody–antigen interactions can help to better understand the binding characteristics of antibodies to antigens, and this can ultimately help improve the affinity and specificity of immunoassays by helping re-design the hapten or optimize ELISA conditions (coating antigen, tracer, pH, ionic strength, etc.). The information can also be used for rational design of recombinant antibodies during genetic manipulation.

3.4. Study of effects on antibody production

Many factors can affect antibody production, such as the selection of adjuvant, the amount of antigen, and the complex response of the immune system [81–89]. The knowledge of the three-dimensional conformation of the antigen is also extremely

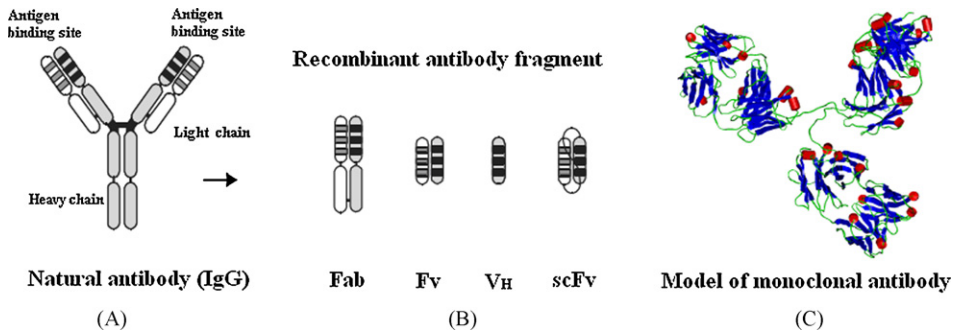

Fig. 4. A schematic diagram illustrating the structure of a conventional antibody molecule (IgG) (A), the different recombinant formats of IgG (B) [78], and the molecular model of a monoclonal antibody (PDB code: 1IGT) (C) [29].

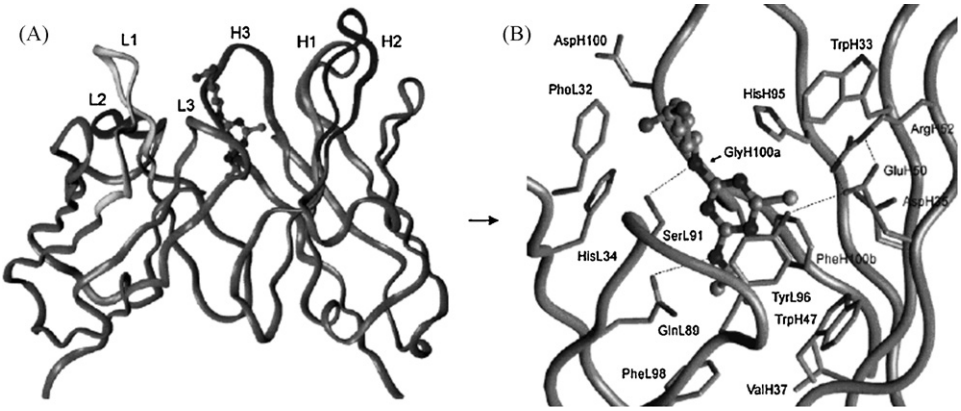

Fig. 5. A ribbon representation of an anti-atrazine antibody fragment (A), and the interactions between the hapten iPr/C1/C6 and the binding site of K4118 (B). The position of the binding site is indicated by a ball representation of the hapten iPr/C1/C6 (A). The side chain residues interacting with hapten iPr/C1/C6 are displayed and labeled, and the predicted hydrogen bonds and salt bridge are shown as dashed lines (B) [37].

important [90,91]. The alteration of a hapten's three-dimensional conformation may have a large impact on antibody production. CAMM has made it possible to evaluate for the first time, antibody production following alteration of the hapten three-dimensional conformation due to increased body temperature of the mouse. Muldoon et al. were able to show that the body temperature of the hapten-conjugate-injected mouse was high-enough above room temperature to rearrange the three-dimensional conformation of the sulfonamide hapten [35]. Twenty-two sulfonamides were evaluated at room temperature for cross-reactivity with the MAb sulfa-1. Two of the sulfonamides, sulfanitran and sulfapyridine, demonstrated much higher cross-reactivity than that of the hapten, while all the other compounds tested had a much lower cross-reactivity. CAMM was used to calculate the minimum energy conformations of these compounds at room temperature, and the observed results did not correlate with the calculated results. Sulfanitran and sulfapyridine models showed a different three-dimensional conformation than did the hapten at room temperature. One would not have expected the observed results based on the calculated three-dimensional conformation at room temperature. Then the relative flexibility of the molecules was studied with respect to temperature by using CAMM methods to calculate potential energy-conformation maps [35]. Fig. 6 shows the potential energy-conformation map of the hapten (SUL) with respect to the energy barriers required to obtain the new three-dimensional conformation, SUL-B. The structure of SUL-B mimics the structure of the two sulfonamides, sulfanitran and sulfapyridine [35]. To achieve the structure of SUL-B, the hapten SUL must go over a potential energy barrier of +3.13 kcal. The structure SUL-B is easily reached at the mouse body temperature, allowing the new structure SUL-B to be presented to the immune system, where antibodies to the

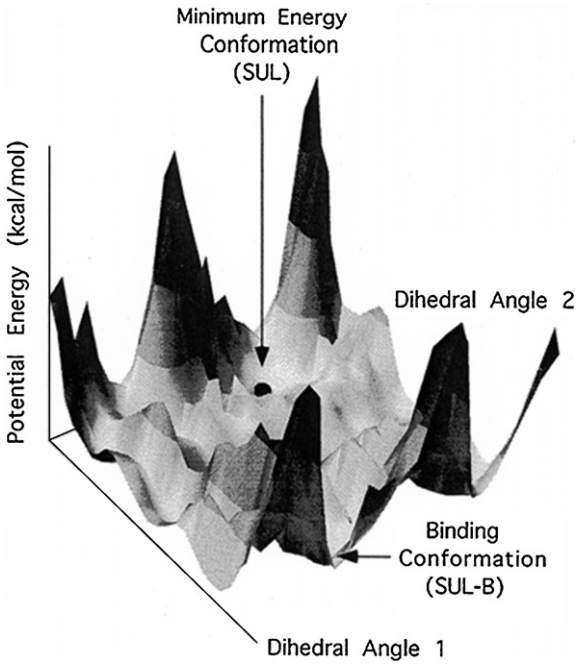

Fig. 6. A potential energy-conformational map derived from the molecular model of the hapten *N*-sulfanilyl-4-aminobenzoic acid (SUL). The long arrow shows where the minimum energy conformation of SUL is at room temperature. The short arrow shows where the new conformation of SUL is after going over the +3.13 kcal mol^{-1} energy barrier [35].

structure of SUL-B could be produced instead of to the desired SUL structure [35]. This phenomenon was observed only because CAMM was used by Muldoon et al. to help them understand their cross-reactivity data [35]. This result may have gone undetected during routine antibody production to small molecules, resulting in a failure to obtain useful antibodies. Certainly, there are plenty of times when one cannot produce antibodies to the selected target. Some of these non-successes may be a result of altering the three-dimensional conformation of the hapten due to increased body temperature of the mouse (or host animal). In cases like these, it might be possible to use CAMM to determine the problematic structural area of the hapten. One could then perform a chemical procedure on the hapten to lock it into the appropriate conformation so that it mimics the conformation of the analyte of interest. Alternatively, one may use a different hapten whose structure is not susceptible to rearrangement by the increased temperature of the mouse.

4. Discussion

4.1. Limitations

Although computational techniques have already provided significant benefits to researchers in designing haptens and evaluating cross-reactivity, it is still an evolving technology and has a number of limitations [28]. Compared with CAMM applications in the field of biology and drug design, the use of CAMM in designing immunoassays has been limited. As of today, only a few reports describing the development of immunoassays with the aid of CAMM have been published. Obviously, the complexity of the animal immune response to the hapten-conjugates used as immunogens can limit the involvement of the researcher. Also, the immunochemist's intuition and training is necessary to interpret the experimental data and results obtained from CAMM. First, the T-cell or B-cell receptor binding site for antigens is unknown, unlike the known binding sites in the drug design field (such as the exact three-dimensional conformation of an enzyme binding site obtained by X-ray analysis), which limits the creativity of the researcher while designing haptens. Many of the researchers cited in this review have designed several haptens based on careful examination of the analyte structure of interest, and then CAMM was used to compare the three-dimensional conformation and electronic properties of the proposed haptens to the authentic analyte. Haptens with similar characteristics to the analyte of interest were chosen to be used during immunization. However, not all results obtained using CAMM were desirable [6,35]. Second, CAMM has been used to explain the cross-reactivity of antibodies; often the results studied were unexpected or undesirable. In most cases, CAMM was used as a qualitative tool to explain the binding characteristics between an antigen and antibody. It can visualize the antibody binding site and help improve the binding ability of an antibody through substrate modification or by site-directed mutagenesis. An overall limitation of using cross-reactivity data as a way of evaluating the binding of various chemicals and haptens with respect to the analyte of interest is that this data is always obtained using an ELISA, which is markedly affected by the competitor (the coating antigen) and the components used in the ELISA. Third, molecular modeling of an antibody binding site (such as found in the scFv or Fab fragments) and its binding interactions with a hapten is possible only when the amino acid sequence is known [92]. Also, few useful recombinant antibodies that recognize small molecular food contaminants have been produced [25]. It is understood that CAMM studies of proteins are limited by the availability of accurate three-dimensional protein structures for comparison [66], which can be obtained by X-ray techniques.

4.2. Prospects

Although the use of immunoassays has become well established, the further development of immunoassay techniques will highly be dependant on the capacity to develop antibodies in a more rapid and economic manner. For many immunoassays, the design and synthesis of the hapten is one of the most time-consuming steps of the antibody production process, and the rationality of these approaches will govern the analytical capability of the immunoassay. Therefore, the synthetic effort necessary to produce antibodies against small molecules should be carefully evaluated in relation to the chances of obtaining a good antibody. CAMM is a tool that can improve the chance of obtaining a good antibody. CAMM methods are able to investigate aspects of the three-dimensional conformation, hydrophobic and electronic properties, hydrogen bonding and Van der Waals forces, and therefore are very useful in hapten design, either in a predictive manner or as a tool to help interpret cross-reactivity studies.

Based on cross-reactivity of antibodies, and the three-dimensional conformation, hydrophobic and electronic properties of the target analyte(s) and its analogs, information about what structural and electronic features important for antibody binding can be obtained. CAMM not only can be useful in an iterative process producing feed-back information that can help improve hapten design, but it can also be useful in altering the ELISA assay format to improve assay performance. Studies that employed heterologous ELISA formats for development of specific assays for ceftiofur [31], nicarbazin [13], 2,4,6-trichlorophenol [93], and a group-specific assay for sulfonamides [94] were highly dependent on information gleaned from CAMM studies carried out on the targets of concern. It was determined that when heterologous ELISA formats were used in combination with CAMM studies, it was possible to improve assay performance in a more rapid manner.

Structural information resulting from molecular interactions between an antibody and antigen can help in understanding the effect of mutations on the affinity and specificity of the antibody [95]. Antibodies with improved affinity can be generated by increasing the number of favorable interactions at the antibody–antigen interface [96]. However, a detailed interpretation of mutational effects on affinity requires precise high-resolution structures, which can be obtained by using X-ray crystallography, but are often not available due to the difficulty and expense of X-ray crystallography. CAMM studies of the molecule and/or system can yield useful information on which to base directed mutagenesis. The benefits of rapid model generation techniques may lead to hypotheses that can be quickly tested, while providing a cost-effective option. For example, the use of CAMM for evaluation of the interactions between a scFv antibody and parathion provides an appropriate illustration [45]. As recombinant antibody technology for low molecular weight food contaminants develops, the application of CAMM in constructing antibody models based on sequence homology and docking of hapten/antigens with antibody models will be useful for improving the properties of recombinant antibodies by site-directed mutagenesis.

5. Conclusions

Computer-assisted molecular modeling is a relatively new area of science that can assist in the development of immunoassays for low molecular weight compounds. CAMM is a tool that can help provide insights into three-dimensional molecular structure and biological activity that are difficult or impossible to obtain in any other way. CAMM can provide important information useful in hapten design, explaining cross-reactivity, and understanding antibody–antigen interactions. The applications of CAMM may help

advance the field of immunological production of highly desired antibodies, and help provide answers to yet unanswered questions, as well as provide insights into future immunological questions. Today, the immunochemist will be more profoundly prepared to answer new challenges in antibody production and immunoassay development by including CAMM methods among their tools.

Acknowledgements

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